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Rational screening of oligonucleotide combinatorial libraries for drug discovery

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ABSTRACT

Combinatorial strategies offer the potential to generate and screen extremely large numbers of compounds and to identify individual molecules with a desired binding specificity or pharmacological activity. We describe a combinatorial strategy for oligonucleotides in which the library is generated and screened without using enzymes. Freedom from enzymes enables the use of oligonucleotide analogues. This dramatically extends the scope of both the compounds and the targets that may be screened. We demonstrate the utility of the method by screening 2′-O-Methyl and phosphorothioate oligonucleotide analogue libraries. Compounds have been identified that bind to the activated *H-ras* mRNA and that have potent antiviral activity against the human herpes simplex virus.

INTRODUCTION

Novel oligonucleotide analogues are a new class of chemicals which have potential use as therapeutic, diagnostic or research reagent applications. Recently reported selection and amplification strategies to identify RNA and DNA ligands for specific molecular targets demonstrate that nucleic acid ligands can bind molecules not known to associate with nucleic acids (1,2). However, these strategies utilize the polymerase chain reaction to generate and screen the oligonucleotide libraries, which limits the library to substrates accepted by polymerases. Over 100 nucleotide analogues (3,4) are now available. Methods which permit the use of these analogues in combinatorial libraries enormously expands the sequence space (5) which can be searched. Moreover, because many oligonucleotide analogues are resistant to naturally occurring nucleases, analogue libraries can be screened in biological assays in which natural DNA or RNA is rapidly degraded.

Combinatorial peptide strategies involving iterative synthesis and selection have been used to identify an antigenic determinant recognized by a monoclonal antibody, an antimicrobial peptide and a protease inhibitor (6,7). Here we describe a oligonucleotide combinatorial technique, known as synthetic unrandomization of randomized fragments (SURF), which is based upon repetitive synthesis and screening of increasingly simplified sets of oligonucleotide analogue pools. The starting pools consist of oligonucleotide analogues of defined length with one position in each pool containing a known analogue and the remaining positions containing equimolar mixtures of all other analogues. With each additional step of the method, at least one additional position of the oligomer is determined until the active pharmacophore is uniquely identified. Using this method, we have identified an accessible binding site on a highly structured RNA hairpin loop and a compound which has potent antiviral activity against the human herpes simplex virus.

RESULTS

RNA hairpin binding

The method is illustrated in Table 1, where 2′-O-Methyl oligonucleotide analogue libraries were screened to identify a compound with high affinity for an RNA hairpin from the activated *H-ras* mRNA (Fig. 1). 2-O-Methyl analogues are known to bind RNA with high affinity and are relatively resistant to nuclease degradation (8). The oligonucleotide library initially screened consisted of 262,144 unique sequences in 4 pools of 65,536. Each pool was tested for binding against the RNA target and a 'pool K_D' was determined. The process was repeated for 9 rounds until a unique 'winner' was identified. As illustrated in Table 1, it was not difficult to distinguish the pool with the lowest K_D at each round of synthesis and screening. As expected for oligonucleotide hybridization reactions (9), positions near the center of the oligonucleotide had a greater effect on the K_D than positions on the extreme 5′ or 3′ ends. For example, an attempt to fix the 3′ position in round 4 did not yield results that distinguished the pools. We selected an alternative position for round 4 which yielded a clear winner, and then proceeded to work from the center of the oligomer to the ends. The final oligonucleotide selected by the SURF procedure is complementary to the single stranded loop region of the target RNA. This result demonstrates that the SURF method can be used to find accessible hybridization sites within a highly structured RNA target.

Table 1. Binding affinities for 2'-O-Methyl 9-mer oligonucleotide pools targeted to H-ras mRNA fragment.

Round	Sequence	Q	K_D (μ) when X = A	C	G	U
1	NNNNXNNNN	65,536	22	10	>100	>100
2	NNNNCNXNN	16,384	>10	4	>10	>10
3	NNXNCNCNN	4,096	>10	0.5	>10	>10
4	NNCXCNCNN	1,024	>10	0.15	>10	>10
5	NNCCCXCNN	256	0.08	>1	0.4	>1
6	NNCCCACXN	64	0.05	>0.5	0.08	>0.5
7	NXCCCACAN	16	>0.1	>0.1	0.03	>0.1
8	NGCCCACAX	4	0.05	0.02	0.05	0.042
9	XGCCCACAC	1	0.03	0.05	0.02	0.01

The Q parameter refers to the number of different sequences contained within the pool. K_D's were determined by gel shift analysis (15). Oligonucleotide pools were synthesized and purified as described (16). The millimolar extinction coefficient used to calculate the concentration of random oligonucleotide pools was derived from the near neighbor rules when N = 10.36, A = 11.99, T = 9.65, C = 9.04, and G = 10.81. A range of concentrations were incubated with 50 pM ^{32}P 5'-end labeled RNA in a buffer containing 100 mM Na^+ at 37°C for 2 h. Oligonucleotide/RNA complex was separated from unbound RNA by electrophoresis on a 15% native acrylamide gel with TBE containing 25 mM NaCl and 0.5 mM $MgCl_2$. The dried gel was analyzed using a Molecular Dynamics phosphoimager.

Herpes simplex virus inhibition

Phosphorothioate oligonucleotides represent another type of analogue that is substantially nuclease resistant (10). A library of 65,536 unique 8-mers in 4 pools of 16,348 each was screened for activity against human herpes simplex virus type 1 (HSV-1) in cell culture. As illustrated in Table II, antiviral activity was observed with increasing potency at each round of synthesis and screening, with no difficulty discerning the most active pool in each round. In contrast to the ras RNA target, the oligonucleotide pool containing a fixed guanine had the most activity in every round of HSV screening except the last round, resulting in selection of a guanine at nearly all fixed positions.

A series of specific oligonucleotides containing varying numbers of contiguous G's was screened using both immunoassays and virus yield assays. Several oligonucleotides which contained a G_4 core had potent antiviral activity, suggesting that G_4 is the minimum active pharmacophore. Sequences flanking the G_4 play an important role in antiviral activity because activity can be modulated by substituting or deleting the surrounding sequences and an unflanked G_4 tetramer showed relatively weak antiviral activity (IC_{50}=2.8 μM). To determine the optimal 8-mer containing a G_4 core, a SURF library was designed as shown in Table III. Optimization of the sequences surrounding the G_4 core produced a 3 fold increase in antiviral activity in four rounds of synthesis and screening, suggesting that although the G_4 core is the most important component of the activity, potency can be modulated by the flanking sequences.

The cytotoxicity of phosphorothioate oligonucleotides selected for anti-HSV activity were investigated using MTT reduction as an end point for cell viability (11). Treatment of cells was exactly as performed in antiviral assays except that no virus was added (see Table 2 legend). In this assay, acyclovir (a licensed nucleoside analog with anti-HSV activity (12)) reduced cell viability by 50% at concentrations of approximately 45μM. Neither of the selected oligos, GGGGGGTG (from Table 2), or GCGGGGTA (from Table 3) reduced cell viability to the 50% level at concentrations as high as 100μM. However, some evidence of changes in cell morphology were observed in cells treated with GGGGGGTG at concentrations of 25μM or higher. For this oligonucleotide, slight reductions in cell viability

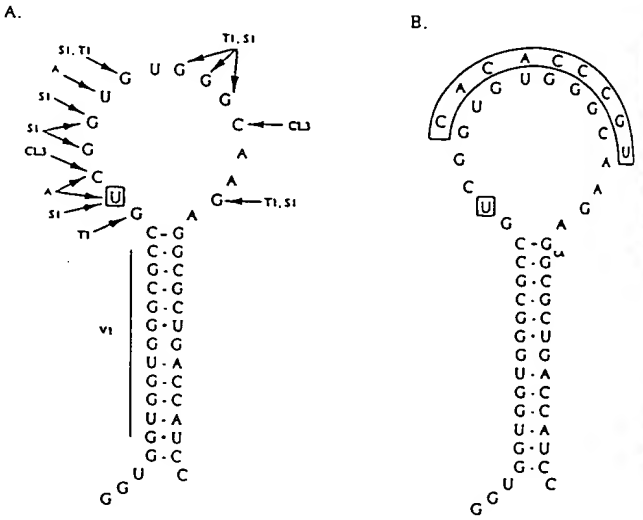

Figure 1. RNA target and binding site for the winning 9-mer oligonucleotide from the SURF procedure. A. The structure for the 47-mer fragment of ras RNA was determined by enzymatic probing (17). RNAse A, RNAse T_1 and Cl_3 specifically cleave single stranded regions; RNAse V_1 cleaves double stranded substrate. B. Binding site for winning 9-mer on the target RNA. The boxed base is the point mutation at codon 12 which is responsible for the gly→val mutation in activated ras.

(70–80%) of untreated cell values) were also noted in this concentration range. For the oligonucleotide GCGGGGTA, no evidence of morphological changes or cell viability was observed at concentrations up to 100μM. These studies confirm that the antiviral activity of the selected oligonucleotides is independent from a cytotoxic effect.

G_4 containing oligonucleotides form a structure known as guanine quartet where four guanines form a cyclic array with the Watson–Crick face and the Hoogsteen face of the guanine involved in hydrogen bonds (13,14). Four sequential G-quartets stack upon each other to form a cylindrical structure with a guanine quadraplex core and a sugar phosphate cylindrical surface. Gel filtration chromatography, CD spectroscopy and NMR characterization of some of the active antiviral sequences showed that the phosphorothioate G_4 containing molecules form

Table 2. Inhibition of human herpes simplex virus 1 by phosphorothioate oligonucleotide pools.

Round	Sequence	Q	IC_{50} (μM) when X = A	C	G	T
1	NNNXNNNN	16,348	>100	>100	70	>100
2	NNNGNNNX	4,096	>100	>100	30	>100
3	NNNGNXNG	1,024	>100	>100	15	>100
4	NXNGNGNG	256	30	30	5	20
5	XGNGNGNG	64	20	20	1.5	20
6	GGNGXGNG	16	10	10	1.5	10
7	GGXGGGGNG	4	1.3	1.3	0.5	1.3
8	GGGGGGXG	1	0.7	0.7	1.1	0.4

ELISA for detection of HSV-1 envelope glycoprotein B (gB) was performed by infection of normal dermal fibroblast cells (NHDF, Clonetics) with HSV-1 (KOS) at a multiplicity of infection of 0.05 PFU/cell. Following virus adsorption for 90 minutes, cells were rinsed one time with culture media (Fibroblast Growth Medium, Clonetics Corp. San Diego) and culture media containing oligonucleotide was added. Cells were fixed 48 h postinfection and assayed for the presence of HSV-1 gB antigen using a monoclonal antibody (Chemicon, MAB8531). Assay results are expressed as the percent of control (infected cells which were not treated with oligonucleotide). Values reported are based on the mean values of triplicate determinations at each concentration. Statistical comparisons using replicates at each concentration revealed significant differences between antiviral activity in the most active pool compared to the other pools in all rounds except for round 8. In round 8, statistically significant differences were not observed between pools where X=T and those where X=A or C.

quartet structures which are related to their antiviral activity (J. Wyatt, unpublished results). Substitution of any of the sequential 4 G's resulted in loss of both the ability of the molecules to form intermolecular quartets and antiviral activity.

DISCUSSION

Some general features of the SURF method are that identification of an active pool in the first round of synthesis and screening always results in identification of at least one unique pharmacophore. Each subsequent set of pools is a subset of the previously selected pool and all molecules in the originally active pool are contained somewhere within the four subsets. Thus, an active molecule contained within the winner pool in the Round 1 screen will be enriched in only one of the next four pools and absent from the other three. There may be several active molecules in the round 1 pool which contribute to the activity observed in the first round screen particularly for targets like RNA, which have more than one potential binding site. Choice of the fixed position might influence the molecule ultimately identified. Testing several different fixed positions could potentially avoid becoming trapped in a 'local minimum', where the most active molecule is not found because selection of the fixed position directed the unrandomization scheme towards a suboptimal winner.

Another important consideration is the size of the pharmacophore and the probability that the active pharmacophore may appear in different places within the same oligonucleotide. In the case of HSV-1, the active pharmacophore was a G_4 core whose activity was modulated by the flanking sequences. Because an 8-mer can contain G_4 in four different places, each new pool containing a G in the variable position had a higher concentration of different G_4 containing oligonucleotides in the pool relative to the other pools. However, when the G_4 core was fixed in the initial library (Table 3), the remaining sequences showed no strong preference for additional G's. Short pharmacophores within longer sequences can be identified by taking the final sequence and systematically substituting each nucleotide position to determine which are essential for retention of activity.

Table 3. Optimization of a G_4 core-containing 8-mer oligonucleotide for HSV antiviral activity.

Sequence	Most Active X =	IC_{50}(μM)
NNGGGGNX	A	2.5
NNGGGGXA	T	1.1
XNGGGGTA	G	0.8
GXGGGGTA	C	0.8

Careful design of screening assays is required for the successful application of SURF technology so that the selected oligonucleotides have the desired specificity. For example, when selecting for antiviral activity, cytotoxicity assays should be performed to insure that inhibition of virus replication is not an artifact of undesirable effects on cell metabolism. It is clear that the antiviral activities of the 2 phosphorothioate oligonucleotides; GGGGGGTG and GCGGGGTA, are unrelated to cytotoxic effects which are observed only at concentrations at least 50 fold greater than those necessary to achieve antiviral activity. Nevertheless, the reduced cytotoxicity (and therefore enhanced therapeutic index) of GCGGGGTA relative to GGGGGGTG suggests that this compound may have advantages as a drug development candidate.

In contrast to peptides, nucleic acids and their analogues are known to fold into three dimensional shapes based upon complex patterns of hydrogen bonding by the heterocyclic bases. These hydrogen bonding patterns provide the basis for shape diversity within a library of linear oligonucleotides. It is interesting that in the two examples described in this paper, the SURF method produced winners based on two different hydrogen bonding motifs, Watson-Crick base pairing (ras) and G-quartets (HSV). A significant advantage of the SURF method is that it enables the screening of oligonucleotide analogues libraries. The libraries described here were composed of uniform sugar (2'-O-Methyl) and phosphate (phosphorothioate) modifications with the common nucleotide bases (A, C, G, U or T). However, base analogues could be used to increase the number of letters resulting in libraries containing a much more complex collection of shapes. The combination of new classes of nucleotide analogues, the

ability to oligomerize them in a controlled fashion and a combinatorial method to screen for activity in complex libraries provides a powerful new vehicle for drug discovery.

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